09/554980

BASIC FIBROBLAST GROWTH FACTOR TREATMENT OF SEPSIS

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BACKGROUND OF THE INVENTION

Federal Funding Legend

This invention was created in part using funds from the National Institutes of Health under grant CA52462. The federal government, therefore, has certain rights in this invention.

Field of the Invention

The present invention relates generally to the fields of medicine and cytokine therapeutics. More specifically, the present invention relates to the basic fibroblast growth factor treatment of septic shock.

Description of the Related Art

Endotoxic shock is a potentially lethal complication of systemic infection by gram-negative bacteria [1, 2]. The toxin

responsible for the induction of endotoxic shock is the glycolipid lipopolysaccharide (LPS), the only lipid present in the outer membrane of gram-negative bacteria. Release of lipopolysaccharide into the circulation activates a series of tissue responses that in their most severe forms lead to septic shock and death. Major events in the pathogenesis of the lipopolysaccharide syndrome include neutrophil, monocyte and macrophage inflammatory responses, intravascular coagulopathy resulting from activation of plasma complement and clotting cascades, endothelial cell damage and hypotension. Death of patients results from extensive tissue injury, multiple organ failure and circulatory collapse.

Although a number of cytokines, including IL-1β, IL-6, and IL-8 are released by lipopolysaccharide-activated inflammatory cells during the onset of the endotoxic response [3], mounting evidence points to TNFα as a primary mediator of this event 4-6]. Not only are substantial quantities of TNFα rapidly released into the circulation, but intravenous injection of TNFα produces a systemic response very similar to lipopolysaccharide. Further, approaches to interfere with TNF action, such as using neutralizing antibodies [4-6] or TNF binding proteins (TNF-bps), abrogate experimental endotoxic shock [7-11] . Perhaps the most compelling evidence for a role for TNFα is the attenuation of endotoxic shock observed in mice lacking the 55 kDa TNF receptor [12, 13].

Although TNFα was originally defined as a cytokine capable of inducing necrosis of tumors *in vivo*, recent studies suggest that in most instances TNFα initiates an apoptotic form of

cell death. In this regard, numerous studies have linked activation of the sphingomyelin pathway to the induction of apoptosis by TNFα. The sphingomyelin pathway is an ubiquitous, evolutionarily conserved signaling system analogous to the cAMP and phosphoinositide pathways. Sphingomyelin (N-acylsphingosin-1-phosphocholine) is a phospholipid preferentially concentrated in the plasma membrane of mammalian cells [14] . Sphingomyelin catabolism occurs via the action of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases, which hydrolyze the phosphodiester bond of sphingomyelin, yielding ceramide and phosphorylcholine. Several forms of sphingomyelinase exist, distinguished by their pH optima [15]. Human and murine acid sphingomyelinase (ASMase; pH optimum 4.5-5.0) have been cloned and determined to be the products of a conserved gene, while Mg^{2+}-dependent or -independent neutral SMases (NSMase) (pH optimum 7.4) have yet to be characterized molecularly. ASMase knock out mice retain NSMase activity, indicating that the neutral forms are products of a distinct gene or genes [16] .

Signaling through the sphingomyelin pathway is mediated via generation of ceramide, which acts as a second messenger in stimulating a variety of cellular functions [17-19]. Receptors distinct as CD28, CD95, and the TNFα, IL-1β, progesterone, γ-interferon and glucocorticoid receptors signal via the sphingomyelin pathway following ligand binding. Thus ceramide signals pleiotropic cellular functions, including proliferation of fibroblasts, differentiation of promyelocytes, inhibition of the respiratory burst in human neutrophils, survival of T9 glioma cells and apoptosis.

Studies on the involvement of the sphingomyelin signaling system in apoptosis revealed that several cytokines and environmental stresses, including TNFα [20-22] , CD95/Fas/APO-1 [23-25 , ionizing radiation, ultraviolet-C, heat and oxidative stress [26-28] induce rapid ceramide generation while leading to an apoptotic response. Further, cell-permeable ceramide analogs, but not analogs of other lipid second messengers, mimicked the effect of cytokines and stress to induce apoptosis. Ceramide action was stereospecific, as analogs of the naturally occurring dihydroceramide, failed to initiate the apoptotic program. These studies suggested that ceramide mediates cytokine- and stress-induced apoptosis.

Definitive evidence for role of ASMase and ceramide in signaling one form of stress-induced apoptosis was derived from studies using genetic models of ASMase deficiency. Santana et al. [29] reported that lymphoblasts from patients with Niemann-Pick disease (NPD), an inherited deficiency of ASMase, manifested defects in ceramide generation and the apoptotic response to ionizing radiation. These abnormalities were reversible upon restoration of ASMase activity by retroviral transfer of human ASMase cDNA. Further, ASMase knockout mice failed to generate ceramide and develop typical apoptotic lesions in the pulmonary endothelium after exposure to total body irradiation. The apoptotic response in the thymus, however, was preserved. The exact opposite occurred in irradiated p53 knockout mice. Whereas the thymus of the p53 knockout mouse was protected against radiation-induced apoptosis, the lung endothelium was not. Differences were observed in other tissues as well. While these studies demonstrated that radiation is capable of activating

two apparently distinct and independent signaling mechanisms for induction of apoptosis, they also suggested a specific sensitivity of endothelial cells towards the ASMase-mediated signaling system for initiating apoptosis in response to stress.

Since both TNFα and endothelial cell damage are critically involved in the pathogenesis of the endotoxic syndrome, it is not known whether ceramide-mediated endothelial cell apoptosis plays a role in the lipopolysaccharide-induced response in vivo. Genetic and pharmacologic manipulations allowed for molecular ordering of the early and critical events in the progression of this syndrome. The prior art is deficient in the lack of effective means of inhibiting the adverse biological effects of TNFα and limiting endothelial cell damage. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a method of inhibiting the generation of ceramide from sphingomyelin comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal in need of such treatment.

In another embodiment of the present invention, there is provided a method of treating a pathophysiological state characterized by endothelial apoptosis, comprising the administration of a pharmacologically effective dose of a basic fibroblast growth factor to said animal.

In yet another embodiment of the present invention, there is provided a method of treating sepsis in an animal in need

of such treatment, comprising the administration of a pharmacologically effective dose of a basic fibroblast growth factor to said animal.

In yet another embodiment of the present invention, there is provided a method of treating an individual at risk for sepsis, comprising the step of administering to said individual a pharmacologically effective dose of a basic fibroblast growth factor.

The endotoxic shock syndrome is characterized by systemic inflammation, multiple organ damage, circulatory collapse and death. Systemic release of tumor necrosis factor-α and other cytokines purportedly mediates this process. However, the primary tissue target remains unidentified. The present invention provides evidence that endotoxic shock results from disseminated endothelial apoptosis. Injection of lipopolysaccharide, and its putative effector TNFα, into $C_{57}BL/6$ mice induced apoptosis in endothelium of intestine, lung, fat and thymus after 6 hours, preceding non-endothelial tissue damage. Lipopolysaccharide or TNFα injection was followed within one hour by tissue generation of the pro-apoptotic lipid ceramide. TNF-binding protein, which protects against lipopolysaccharide-induced death, blocked lipopolysaccharide-induced ceramide generation and endothelial apoptosis, suggesting systemic TNF is required for both responses. Acid sphingomyelinase knockout mice displayed a normal increase in serum TNFα in response to lipopolysaccharide, yet were protected against endothelial apoptosis and animal death, defining a role for ceramide in mediating the endotoxic response. Further, intravenous injection of basic fibroblast growth factor, which acts as an intravascular

survival factor for endothelial cells, blocked lipopolysaccharide-induced ceramide elevation, endothelial apoptosis and animal death, but did not affect lipopolysaccharide-induced elevation of serum TNFα. These investigations demonstrate that lipopolysaccharide induces a disseminated form of endothelial apoptosis, mediated sequentially by TNF and ceramide generation, and suggest that this cascade is mandatory for evolution of the endotoxic syndrome.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows that lipopolysaccharide induces, and TNF-bp blocks, apoptosis in the endothelium of (Figure 1A) intestine, lung, pericolic fat and (B) thymus. $C_{57}BL/6$ mice were injected intraperitoneally with 90 µg of *S. typhimurium*

lipopolysaccharide/25 g of mouse body weight or diluent (PBS), and after 6 hours were sacrificed by hypercapnia asphyxiation. For studies employing TNF-bp, animals were injected with 75 μg of TNF-bp/25 g of mouse body weight or with diluent (PBS) two hours prior to lipopolysaccharide. Tissue specimens were fixed overnight in 4% buffered formaldehyde and apoptosis assessed as in Methods by TUNEL assay (Figure 1A) or a combination of TUNEL and immunohistochemical staining for the cell surface antigen CD31 (Figure 1B). Nuclei of apoptotic cells appear brown and granular, and in (Figure 1B) are surrounded by a blue-black perimeter. Normal nuclei in (Figure 1A) stain blue and in (Figure 1B) stain red due to hematoxylin and fast red counterstains, respectively. Original magnification: intestine x400; lung, pericolic fat and thymus x1000. This experiment represents one of three similar studies.

Figure 2 shows that lipopolysaccharide induces rapid ceramide generation in the mucosa of the intestine. These studies were performed as in Figure 1 except mice were sacrificed at the indicated times. The intestinal mucosa was dissected as described. Mucosal specimens were homogenized in 8 volumes (v/v) of ice-cold PBS and lipids were extracted with 2 ml of chloroform:methanol (2:1, v/v)/400 μl of homogenate. After mild alkaline hydrolysis to remove glycerophospholipids, ceramide was quantified using *E. coli* diacylglycerol kinase (Calbiochem) as described [27], and results normalized for protein content. The data (mean ± S.E.M.) represent triplicate determinations from two mice per point from two experiments for (Figure 2A) time course, and from one representative of two experiments for (Figure 2B) dose-dependence.

Figure 3 shows that TNFα induces ceramide generation in intestinal mucosa. Figure 3A shows the time course of the effect of 25 μg of recombinant human TNFα/25g mouse; (Figure 3B) Dose-response at 2 hours. These studies were performed as in Figure 2 except C_{57}BL/6 mice were injected with TNFα retro-orbitally. The data (mean ± S.E.M.) represent triplicate determinations from two mice per point from one representative of three experiments for Figure 3A and one representative of four experiments for Figure 3B.

Figure 4 shows that TNF-bp blocks lipopolysaccharide-induced ceramide generation. For these studies, animals were injected with TNF-bp and lipopolysaccharide as described in Figure 1, and ceramide levels determined as in Figure 2. These data (mean ± S.E.M.) represent triplicate determinations from two mice per point from two experiments.

Figure 5 shows that the acid SMase knock-out mice are defective in lipopolysaccharide-induced death. Actuarial (Kaplan-Meier) survival curves of wild type and ASMase knockout mice injected intraperitoneally with 175 μg of lipopolysaccharide/25g mouse. The number in parenthesis indicates the number of animals in each group.

Figure 6 shows that basic FGF blocks lipopolysaccharide-induced endothelial apoptosis and animal death. Figure 6A shows that C_{57}BL/6 mice injected intravenously with 800 ng bFGF 30 min prior to, and 5 min, 1 and 2 hours after, an intraperitoneal injection of 175 μg of lipopolysaccharide/25g mouse. Endothelial apoptosis was assessed as in Figure 1 by TUNEL assay. Figure 6B shows the actuarial (Kaplan-Meier)

survival curves of $C_{57}BL/6$ treated as in Figure 6A. The number in parenthesis indicates the number of animals in each group.

Figure 7 shows the proposed schema for progression of the endotoxic response. Lipopolysaccharide, released by gram negative bacteria, interacts with inflammatory cells leading to generation of TNFα and other cytokines. TNFα, acting upon endothelium, stimulates sphingomyelin hydrolysis to ceramide, presumably via an ASMase, which then serves as a second messenger for apoptosis. Apoptosis of the endothelium ensues, which can be blocked by bFGF via inhibition of ceramide generation. Endothelial apoptosis may result in generalized microvascular dysfunction sufficient to compromise the circulation to major organs, leading to non-endothelial tissue damage, circulatory collapse, and death.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, injection of lipopolysaccharide into $C_{57}BL/6$ mice resulted in a disseminated form of microvascular endothelial apoptosis, mediated sequentially by TNF and ceramide generation, and suggested that this cascade plays a mandatory role in the evolution of lipopolysaccharide-induced death.

The present invention provides a method of decreasing endothelial ceramide generation from sphingomyelin comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal. Generally, the methods of the present invention are equally advantageous and of desirable use in treating various animals, including mammals.

Most preferably, the methods of the present invention would be most useful in a human.

In a separate embodiment, the present invention pertains to a method of treating a pathophysiological state in animals characterized by endothelial cell death, endogenous levels of senescence inducing proteins, comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal.

A pharmaceutical composition, comprising basic fibroblast growth factor and a pharmaceutically acceptable carrier is also provided. The pharmaceutical compositions of the present invention are suitable for use in a variety of drug delivery systems. For a review of present methods for drug delivery, see Langer, *Science*, 249:1527-1533 (1990). Methods for preparing such compounds will be known or apparent to those skilled in the art and are described in more detail, for example, in Remington's *Pharmaceutical Science*, 17th ed., Mack Publishing Company, Easton, PA (1988). A person having ordinary skill in this art would readily recognize the most appropriate route of administration and dosages for fibroblast growth factor. Preferably, basic fibroblast growth factor is administered in a daily amount of from about 0.01 mg/kg to about 100 mg/kg. The basic fibroblast growth factor may be administered most desirably to an individual at risk for septic shock or its sequalae in a prophylactic fashion.

Basic fibroblast growth factor, pharmaceutically acceptable salt thereof and pharmaceutical compositions incorporating such, may be conveniently administered by any of the routes conventionally used for drug administration, e.g., orally,

topically, parenterally, or by inhalation. Conventional dosage forms can be prepared by combining basic fibroblast growth factor with standard pharmaceutical carriers according to conventional procedures. Basic fibroblast growth factor may also be administered in conventional dosages in combination with a known, second therapeutically active compound. These procedures may involve mixing, granulating and compressing or dissolving the ingredients as appropriate to the desired preparation. It will be appreciated that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of adminstration and other well known variable. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The pharmaceutical carrier employed may be, for example, either a solid or a liquid. Representative solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium sterate, stearic acid and the like. Representative liquid carriers include syrup, peanut oil, olive oil, water and the like. Similarly, the carrier may include time delay material well known in the art such as glyceryl monosterate or glyceryl disterarate alone or with a wax.

A wide variety of pharmaceutical forms can be employed. Thus, if a solid carrier is used, the preparation can be tableted, placed in a hard gelatin capsule in powder or pellet form or in the form of a troche or lozenge. The amount of solid carrier will vary widely but preferably will be from about 25 mg to about 1 gram. When a liquid carrier is used, the preparation will be in

the form of a syrup, emulsion, soft gelatin capsule, sterile injectable liquid such as an ampule or nonaqueous liquid suspension. For all methods of use of the present invention disclosed herein for basic fibroblast growth factor, it will also be recognized by one of skill in this art that the optimal quantity and spacing of individual dosages of a compound of the present invention, or a pharmaceutically acceptable salt thereof, will be determined by the nature and extent of the condition being treated and that such optimums can be determined by conventional techniques.

Suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of inorganic and organic acids, such as hydrochloric acid, hydrobromic acid, sulphuric acid, phophoric acid, methane sulphonic acid, ethane sulphonic acid, acetic acid, malic acid, tartaric acid, citric acid, lactic acid, oxalic acid, succinic acid, fumaric acid, maleic acid, benzoic acid, salicylic acid, phenylacetic acid and mandelic acid. In addition, pharmaceutically acceptable salts of basic fibroblast growth factor may also be formed with a pharmaceutically acceptable cation, for instance, if a substituent group comprises a carboxy moiety. Suitable pharmaceutically acceptable cations are well known in the art and include alkaline, alkaline earth ammonium and quaternary ammonium cations.

Administration of basic fibroblast growth factor in the methods of the present invention may be by topical, parenteral, oral, intranasal, intravenous, intramuscular, subcutaneous, or any other suitable means. The preferred method of administration is by intravenous injection.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

lipopolysaccharide treatment

Using a 26 gauge needle, $C_{57}BL/6$ mice were injected intraperitoneally with lipopolysaccharide (*Salmonella typhimurium*, Westphal purified, Difco Laboratories, MI) resuspended in sterile water. For TNFα and TNF-bp injections, mice were first anesthetized with pentobarbital (50 mg/kg) intraperitoneally. After obtaining adequate anesthesia, recombinant human TNFα or TNF-bp (Amgen, Boulder) was injected intravenously with a 28 gauge needle via a retro-orbital approach. Sham injected animals received diluent. For studies measuring survival, animals were monitored for up to two weeks. Survival as the end point in these experiments was calculated from the time of treatment using the product limit Kaplan-Meier method [30]. Calculations of the dose leading to 50% lethality (LD_{50}) at a given time after lipopolysaccharide treatment was performed using probit analysis. For studies evaluating histology or tissue ceramide content, mice were sacrificed by hypercapnia asphyxiation.

Mice were housed in a pathogen-free environment in the animal facility. This facility is approved by the American Association for Accreditation of Laboratory Animal Care and is maintained in accordance with the regulations and standards of the United States Department of Agriculture and the Department of Health and Human Services, National Institutes of Health.

Lipid Studies

For studies measuring tissue ceramide levels, abdominal contents of sacrificed animals were immediately exposed through a midline incision, and the gastric pylorus was identified. The duodenum was transected and the proximal 3 to 4 centimeter of small intestine were excised and placed on ice. Using a Nikon SMZ-2B dissecting microscope set at 10X magnification, the anti-mesenteric border of the bowel was incised, exposing the mucosal surface of the bowel. The bowel was irrigated with cold phospho-buffered saline (PBS) and the mucosa bluntly dissected from the underlying muscularis propria with curved tissue forceps. Mucosa were homogenized in 8 vol (v/v) of ice-cold PBS. Homogenate (0.6 ml) was transferred to 16x100 mm glass tubes and lipids were extracted with 3 ml of chloroform:methanol (2:1,v/v). After mild alkaline hydrolysis to remove glycerophospholipids ceramide was quantified using *E. coli* diacylglycerol kinase (Calbiochem) as described [27].

Apoptosis

Apoptosis *in vivo* was assessed by the DNA terminal transferase nick-end translation method (also termed the TUNEL assay), as described [31]. Briefly, tissue specimens were fixed overnight in 4% buffered formaldehyde and embedded in paraffin blocks. Tissue sections (5 μm thick), adherent to polylysine-treated slides, were deparaffinized by heating at 90°C for 10 minutes and then at 60°C for 5 minutes. Tissue-mounted slides were first washed with 90% and then 80% ethanol (3 minutes each) and rehydrated. The slides were incubated in 10 mM Tris-HCl, pH 8 for 5 minutes, digested with 0.1% pepsin, rinsed in

distilled water and treated with 3% H_2O_2 in PBS for 5 minutes at 22°C to inactivate endogenous peroxidase. After 3 washes in PBS, the slides were incubated for 15 min at 22°C in buffer (140 mM Na-cacodylate, pH 7.2, 30 mM Trizma base, 1 mM $CoCl_2$) and then for 30 minutes at 37°C in reaction mixture (0.2 U/µl terminal deoxynucleotidyl transferase, 2 nM biotin-11-dUTP, 100 mM Na-cacodylate, pH 7.0, 0.1 mM DTT, 0.05 mg/ml bovine serum albumin, and 2.5 mM $CoCl_2$). The reaction was stopped by transferring the slices to a bath of 300 mM NaCl, 30 mM Na citrate for 15 minutes at 22°C. The slides were washed in PBS, blocked with 2% human serum albumin in PBS for 10 minutes, re-washed and incubated with avidin-biotin peroxidase complexes. After 30 minutes at 22°C, cells were stained with the chromogen 3,3'diamonobenzidine tetrachloride and counterstained with hematoxylin. Nuclei of apoptotic cells appear brown and granular, while normal nuclei stain blue.

Some studies employed double staining with TUNEL to assess apoptosis followed by immunostaining with a rat monoclonal anti-CD 31 antibody to identify endothelial cells. For these studies, TUNEL stained sections were incubated with normal rabbit serum (10% in PBS-bovine serum albumin) (Cappel) and subsequently with a primary rat anti-CD31 antibody at 4°C (1:500 dilution) (Pharmingen). A rat monoclonal antibody of the same subclass as the primary antibody was used as a negative control at a similar working dilution. Biotinylated rabbit anti-rat antibodies were applied for 1 hour (Vector Laboratories, Burlingame, CA - 1:100 dilution), followed by avidin-biotin peroxidase complexes for 30 minutes (Vector Laboratories - 1:25

dilution). True Blue Peroxidase substrate was used as the final chromogen (KPL Laboratories) and nuclear fast red (Vector Laboratories) was used as the nuclear counterstain. Cell surface immunoreactivity was identified as a dark blue staining. Double staining was considered positive when specific cells displayed a brown nuclear stain in the context of a surrounding or superimposed blue-to-black membrane immunoreactive pattern. The scoring of stained tissue was conducted independently by two investigators. The areas scored were always selected randomly and counts by each of the investigators were carried out in a blinded fashion, unveiling the code at the end of the study. Blood was obtained from anesthetized mice through an abdominal incision by aspiration from the inferior vena cava using a 28 gauge needle (Becton Dickinson, Rutherford, NJ). Serum TNFα levels were measured by ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA). Statistical analysis were performed by Student's test and Chi Square test. Differences in product limit Kaplan Meier survival curves were evaluated by the Mantel log-rank test for censored data [32].

Results

Initial studies examined the time course and dose-dependence of lipopolysaccharide-induced death of $C_{57}BL/6$ mice. For these studies, *S. typhimurium* lipopolysaccharide or diluent were injected intraperitoneally. Death was detected as early as 16 hours after a maximal dose of lipopolysaccharide (270 μg/25g mouse) and all of the mice were dead after 48 hours. As little as

60 μg of lipopolysaccharide/25g mouse was effective and the LD_{50} was approximately 90 μg of lipopolysaccharide/25g mouse.

To explore whether endothelial cell apoptosis is associated with the lipopolysaccharide response, $C_{57}BL/6$ mice were injected with 90 μg of lipopolysaccharide/25 g of mouse body weight and multiple tissues were evaluated for an apoptotic response using the TUNEL method. Figure 1 shows that lipopolysaccharide induced an apoptotic response in microvascular endothelial cells of intestinal crypts, the lung, pericolic fat and thymus. Crypts of the intestinal mucosa are comprised of a layer of columnar epithelial cells on the intestinal luminal surface and a central network of capillaries in the lamina propria. Intestinal crypts from sham injected animals demonstrated minimal apoptosis (Figure 1A, left panel). Apoptotic cells display an intense brown nuclear stain, whereas the nuclei of unaffected cells are visualized blue due to the hematoxylin counterstaining. Lipopolysaccharide-injected animals, however, demonstrated diffuse endothelial apoptosis with little if any changes in the epithelial cell layer (Figure 1A, middle panel). This effect was maximal at 6 hours and preceded the onset of apoptosis in the epithelial cells of the crypt, which became apparent after 8-10 hours (data not shown). Similarly, the lungs of sham-treated animals displayed little apoptosis in either capillary endothelial cells or in tissue pneumocytes (Figure 1A, left panel). Substantial and selective apoptotic damage was detected, however, in the pulmonary microvascular endothelium in response to lipopolysaccharide injection by 6 hours (Figure 1A, middle panel). In both these tissues, hematoxylin and eosin stained sections from lipopolysaccharide-treated animals revealed large numbers of

endothelial cells with shrunken pyncnotic nuclei, many of which were fragmented (data not shown). These apoptotic cells appeared to be phagocytized by neighboring cells in some sections. Apoptotic damage to the endothelium of pericolic fat tissue was similarly detected by 6-8 hours after lipopolysaccharide injection, while adipocytes and fibroblasts, seen on the periphery of Figure 1A middle panel, were spared. This effect was also observed in mediastinal and subcutaneous fat tissue (data not shown). In all of these organs, the extent of endothelial, and the subsequent non-endothelial, tissue damage was dose-dependent, increasing from 60 to 175 µg of lipopolysaccharide/25 g of mouse body weight.

Apoptosis was also observed in thymic tissue by 6 hours after lipopolysaccharide injection. Apoptotic cells, as assessed by the TUNEL assay, appeared in the thymus as discrete foci manifesting a configuration reminiscent of a vascular formation. However, the dense packing of cells within this tissue precluded histologic and morphologic identification of the apoptotic cells as endothelium. To determine whether these apoptotic cells were of endothelial origin, a double staining technique was developed. Thymic tissue, stained by the TUNEL method to detect apoptotic nuclei, were co-stained immunohistochemically with an antibody to the endothelial cell surface antigen CD31, also known as platelet endothelial cell adhesion molecule (PECAM)-1 [33]. Normal endothelium of thymic microvessels were identified by dark blue staining of the cell membrane, whereas thymocytes lacked this stain and manifested only a light red nuclear color resulting from the use of fast red as counterstain (Figure 1B, left panel). In specimens from lipopolysaccharide-treated mice, apoptotic endothelial cells

displayed a central brown nuclear core surrounded by a blue-black perimeter (Figure 1B, middle panel). It should be noted that the microvessels identified in the thymus are comprised of only two to four endothelial cells and thus appear smaller than those in the lung and fat, which were frequently comprised of five to eight endothelial cells. Using this double staining technique, virtually all of the apoptotic cells present in the thymus at 6 hours after lipopolysaccharide stimulation represented endothelial cells in microvessels, the lumens of which were partially or completely collapsed (Figure 1B, middle panel). Endothelial apoptosis occurred in all of these tissues in the absence of an inflammatory response, which was subsequently detected at 10-12 hours. Taken together, these studies indicate that intraperitoneal injection of lipopolysaccharide induces a disseminated form of endothelial apoptosis, which precedes non-endothelial parenchymal tissue damage.

The extent of microvascular involvement was quantified. Table I shows that 71% of the intestinal villae and 64-79% of the microvessels of the lung, fat and thymus displayed apoptotic damage at 6 hours after a dose of 90 μg of lipopolysaccharide/25g mouse. Similar effects were observed at 8 hours after injection of 90 μg of lipopolysaccharide/25g mouse and with 175 μg of lipopolysaccharide/25g mouse. Apoptosis was detected in less than 5% of microvessels in tissues from control sham-treated animals.

TABLE I

Quantitation of microvascular apoptosis after lipopolysaccharide and inhibition by TNF-bp

	Apoptotic Blood Vessels	
Tissue	LPS	LPS + TNF bp
Intestine	107 (71%)	13 (8%)*
Lung	104 (70%)	12 (8%)*
Fat	118 (79%)	32 (21%)*
Thymus	95 (64%)	20 (13%)*

* p<0.001 vs. LPS alone

Tissues, obtained from mice treated as in Figure 1, were analyzed for the extent of apoptosis. One hundred and fifty intestinal villae or capillaries from lung, fat or thymus, were scored for apoptosis. Data are presented as the number of positive villae or vessels, and the percentage positively is shown in parentheses.

To determine whether ceramide generation plays a role in lipopolysaccharide-induced apoptosis, $C_{57}BL/6$ mice were treated with 175 μg of lipopolysaccharide/25g mouse and at various periods of time thereafter, the intestinal mucosa was dissected away from the muscularis layer. Ceramide content of

the intestinal mucosa significantly increased from a basal level of 1200 pmol/mg tissue by 1 hour after lipopolysaccharide injection and peaked at 2-fold by 2 hours ($p < 0.001$ vs. control) (Figure 2A). As little as 60 µg/25g mouse was effective and a maximal effect occurred with 175 µg/25g mouse (Figure 2B). Similar ceramide elevation was detected in the lung of C_{57}BL/6 mice within the first hour after lipopolysaccharide injection (n=3). In contrast, the level of the lipid second messenger 1,2-diacylglycerol was not elevated. These studies demonstrate that ceramide generation precedes the apoptotic response.

Since TNFα is a primary mediator of the septic shock response to lipopolysaccharide [4-6] , and since ceramide has been described as a mediator of TNF-induced apoptosis in numerous cellular systems [17-19], the effect of TNFα on tissue ceramide generation, endothelial apoptosis, and survival of C_{57}BL/6 mice was investigated. Recombinant human TNFα, when injected intravenously, induced time- and dose-dependent lethality in this strain of mice. As little as 5 µg of TNFα/25g mouse was effective and the LD_{50}, although somewhat variable between experiments, ranged from 25-50 µg of TNFα/25g mouse. At a dose of 25 µg of TNFα/25g mouse, death occurred as early as 10 hours after injection and the mean time until death in multiple experiments was 24 hours.

Figure 3A shows that TNFα induced time- and dose-dependent ceramide generation in the intestinal mucosa. 25 µg of TNFα/25g mouse stimulated an increase in ceramide content with a slightly more rapid time course than induced by lipopolysaccharide. TNFa-induced ceramide generation was

detected by 0.5 hours and peaked at 1.5 hours ($p < 0.001$ vs. control). As little as 2.5 μg of TNFα/25g mouse was effective and a maximal effect occurred with 25 μg of TNFα/25g mouse (Figure 3B). TNFα, like lipopolysaccharide, induced endothelial apoptosis in intestinal mucosa, lung and fat tissues, beginning 6 hours after injection (data not shown). These studies demonstrate that TNFα, like lipopolysaccharide, induces ceramide generation followed by microvascular endothelial apoptosis and demise of the animal.

Agents that inhibit TNFα action have been shown to prevent the endotoxic shock response in a variety of different experimental models. These include neutralizing antibodies to TNFα [4-6], chimeric inhibitors comprised of the extracellular domain of the TNF receptor fused with an immunoglobulin heavy chain fragment [10] or as a polyethylene glycol-linked dimer (TNF-bp) [9, 11, 34], and a TNF convertase metalloproteinase inhibitor [35], to list a few. To evaluate whether the effect of TNFα to induce tissue ceramide generation and endothelial apoptosis is essential for the lipopolysaccharide effect, TNF-bp was injected with lipopolysaccharide.

Figure 4 shows that intravenous injection of TNF-bp (serum $t_{1/2}$~30 hours) abolished the effect of a maximal dose of 175 μg of lipopolysaccharide/25g mouse on ceramide generation in the intestinal mucosa. Further, TNF-bp markedly attenuated lipopolysaccharide-induced apoptosis in the endothelium of the intestine, lung, pericolic fat and thymic tissue at 6 hours (Figure 1, right panels) and at 8-10 hours (data not shown) after stimulation. Quantitation of apoptotic microvessels in tissues treated with TNF-bp and lipopolysaccharide, demonstrated near complete protection

from apoptosis in all tissues (Table I)($p<0.001$ vs. lipopolysaccharide-treated for each tissue). These studies provide evidence that lipopolysaccharide-induced ceramide generation, endothelial apoptosis, and endotoxic death require TNFα action.

To determine whether ceramide generation is necessary for progression of the endotoxic syndrome, wild type and ASMase knockout mice were treated with 175 μg of lipopolysaccharide/25g mouse. Lipopolysaccharide-induced elevation of serum TNFα was unaffected in the ASMase knockout mice, increasing to a maximum of 12 ± 3 ng/ml at 1.5 hours after lipopolysaccharide injection. These data indicate that monocyte/macrophage activation is normal in the ASMase mouse. However, ASMase knockout mice were defective in lipopolysaccharide-induced ceramide generation and endothelial apoptosis. In contrast to the 2-fold maximal ceramide elevation observed in the intestines of wild type animals 2 hours after 175 μg of lipopolysaccharide/25 g mouse, in the ASMase knockout mouse the ceramide level did not increase significantly and after 2 hours was only 1.27±0.18-fold of control (mean±S.D.; 4 mice/group). Further, upon evaluation of 150 intestinal villae for apoptotic microvessels at 6 hours after injection of 175 μg of lipopolysaccharide/25g mouse, 118 (79%) were positive in the wild type mice, while only 17 (11%) were positive in the ASMase knockout mice ($p<0.001$ vs. lipopolysaccharide-treated wild type; 6 mice/group). Upon evaluation of 150 capillaries in lungs from the same animals, 109 (72%) demonstrated apoptotic damage in the wild type animal, whereas only 15 (10%) were apoptotic in the ASMase knockout mice ($p<0.001$ vs. lipopolysaccharide-treated

wild type). Apoptosis progressed with time in lipopolysaccharide-treated ASMase knockout mice, reaching a maximum at 8 hours when 33 of 150 (22%) intestinal villae, and 29 of 150 (19%) lung microvessels, were positive ($p<0.001$ vs. lipopolysaccharide-treated wild type mice tissues in which 70-80% of microvessels display apoptosis at 8 hours). Further, ASMase knockout mice were protected against lipopolysaccharide-induced death ($p=0.05$ vs. lipopolysaccharide-treated wild type; Figure 5). These studies suggest that lipopolysaccharide-induced apoptosis, like radiation-induced apoptosis, requires a functional sphingomyelin pathway.

To provide additional support for the notion that endothelial damage is essential for evolution of the endotoxic response, $C_{57}BL/6$ mice were treated concomitantly with lipopolysaccharide and bFGF. Prior studies showed that bFGF protected endothelium in vitro and in vivo from radiation-induced apoptosis [31]. In vivo, intravenously injected bFGF has been shown to be retained within blood vessels, apparently bound to the heparan sulfate proteoglycan coating the vascular surface of the endothelium and its basement membrane [31]. Consequently, intravenously injected bFGF served as a selective endothelial survival factor, preventing radiation-induced apoptosis and lethal radiation pneumonitis [36].

Figure 6A shows that bFGF abrogated lipopolysaccharide-induced apoptosis in the endothelium of the intestine and lung of $C_{57}BL/6$ mice. Figure 6B demonstrates that intravenous bFGF, when injected concomitantly with a dose of 175 µg of lipopolysaccharide/25g mouse, provided protection from the lethal effects of lipopolysaccharide ($p<0.001$ vs. untreated). bFGF also rescued $C_{57}BL/6$ mice from maximal doses of 270 and 350 µg

of lipopolysaccharide/25g mouse, although the protection was not as complete (data not shown).

Additional studies delineated the site of bFGF action. Table II shows that while TNFα was not detected in the serum of sham- or bFGF-injected animals, 175 μg of lipopolysaccharide/25g mouse induced an elevation of serum TNFα to a maximum of 4.2 ± 0.9 ng/ml. The lipopolysaccharide-induced elevation of serum TNFα was not blocked by bFGF. In contrast, bFGF prevented the elevation of tissue ceramide in response to lipopolysaccharide. These studies indicate that intravenous bFGF does not affect cell types that generate TNFα in response to lipopolysaccharide (i.e. monocytes and macrophages), but specifically targets endothelial cells and the ceramide response to TNF stimulation. These data also substantiate endothelial damage as mandatory for lipopolysaccharide-induced death, and define inhibition of TNF signaling as the mechanism of the pro
tective effect of bFGF on endothelium.

Table II - Basic FGF blocks LPS-induced tissue ceramide generation but not serum TNFα elevation

	Control	bFGF	LPS	LPS + bFGF
TNFα (ng/ml)	n.d.	n.d.	4.2±0.9	7.9 ±2.0
Ceramide (pmol/µg prot)	1942±97	1352±113	3175±274*	1810±108

n.d. - not detected
* p<0.01 vs. LPS + bFGF and control

Blood and intestinal mucosa were obtained from anesthetized mice (3 per group) 1.5 hours after intraperitoneal injection of 175 μg of lipopolysaccharide/25g mouse with or without a single intravenous injection of 800 ng bFGF, as in Figure 6A. Serum TNFa levels were measured in triplicate by ELISA as described. Ceramide content was measured in triplicate as described in Figure 2.

The present studies define a set of early biochemical and biological responses to lipopolysaccharide using a standard model of endotoxic shock. Figure 7 orders these events. Within 1 hour of intraperitoneal injection of lipopolysaccharide, elevation of tissue ceramide content was detected in the intestinal mucosa and lung. Although this evidence supports endothelium as the primary source of the increase in ceramide, it is possible that cells other than endothelium contribute to the ceramide elevation. Ceramide elevation appeared dependent on TNF action since TNF mimicked the lipopolysaccharide effect, and TNF-bp blocked the lipopolysaccharide-induced increase in tissue ceramide. Elevation of ceramide preceded the appearance of a generalized form of apoptosis, expressed initially in the microvascular endothelium of a variety of organs, beginning at 6 hours after lipopolysaccharide injection. Both ceramide elevation and endothelial apoptosis preceded damage to non-endothelial parenchymal tissue and the death of the animal, which became apparent at 16 hours after a dose of 175 μg lipopolysaccharide/25g mouse. Endothelial apoptosis appeared mandatory for the progression of the endotoxic response, since intravenous injection of bFGF, which specifically protects the endothelium against stress-induced

apoptosis, prevented death. Further, ceramide appeared to be a key intracellular mediator of this response, as the ASMase knockout mouse, which is defective in ceramide generation but not in TNFα production, exhibited decreased endothelial apoptosis and death.

Lipopolysaccharide induces endothelial damage *in vivo* and under some conditions *in vitro*. Microvascular injury occurs in numerous tissues during sepsis, including the lung, gut and liver, and this event has been generally considered an important element in the pathogenesis of the septic shock syndrome [1] . The mechanism of microvascular injury and its relevance to the evolution of the septic shock syndrome have been a subject of substantial debate. Disseminated intravascular thrombosis, extensive endothelial necrosis and humoral microvascular dysfunction have all been ascribed a role as mediating vascular collapse [1]. Generalized endothelial apoptosis has not hitherto been reported, although apoptosis of liver endothelium *ex vivo* was recognized subsequent to induction of TNFα on Kupfer cells by lipopolysaccharide [37]. The large majority of studies reported that lipopolysaccharide did not induce apoptosis in primary cultures of endothelial cells [37-40] unless a second stress such as heat shock or cycloheximide was applied subsequently [39, 40] . One group, however, has argued that lipopolysaccharide can induce direct DNA damage leading to apoptosis in primary cultures of sheep pulmonary endothelial cells [41, 42].

In the present invention, endothelial apoptosis appeared to be preferentially increased in tissues known to play prominent roles in the pathogenesis of endotoxic shock. In this regard, the microvascular endothelium of the bowel and lung were

markedly affected. However, even the endothelium of tissues which play no overt role in the endotoxic response, such as the pericolic fat and the thymus, seemed to be affected. Endothelial damage preceded non-endothelial damage suggesting that loss of vascular integrity may play a role in the parenchymal tissue damage and the multi-organ failure that characterizes the endotoxic syndrome. The generalized nature of the apoptotic response in the microvascular endothelium may account, in part, for the circulatory failure that is a major factor in the progression of the endotoxic response. Whether endothelial apoptosis in the lung is the critical lesion leading to the asphyxiation that results in the ultimate demise of affected mice [5] cannot be ascertained.

The critical role of endothelial cell apoptosis in the pathogenesis of endotoxic shock is similar to its role in the evolution of the inflammatory phase of radiation-induced pneumonitis. As in the case of the lipopolysaccharide response, microvascular endothelial apoptosis preceded the expression of other histopathological manifestations of lethal radiation-induced pneumonitis, and intravenous injections of bFGF abrogated the evolution of pneumonitis and death after whole lung irradiation [31, 36]. Further, both lipopolysaccharide- and radiation-induced endothelial apoptosis *in vivo* appeared initiated by activation of ASMase. Thus, the sphingomyelin pathway may integrate diverse responses to signal death in stressed endothelial cells. Consistent with this hypothesis, the prevention of ceramide generation by bFGF suggests that the anti-apoptotic survival function of bFGF may be mediated, in part, via this mechanism.

The present investigations establish a role for TNFα in lipopolysaccharide-induced generation of ceramide and apoptosis

in vivo. Recent studies have clarified the mechanism by which the 55 kDa TNF receptor signals the apoptotic response [43-52]. This receptor contains a carboxy-terminal death domain which appears to be required for transmission of the apoptotic signal. Binding of TNFα to the receptor triggers formation of a multiprotein complex in which cytoplasmic proteins and the receptor interact through their respective death domain motifs. Upon TNF stimulation, the receptor death domain binds to the death domain of a cytoplasmic protein known as TRADD (TNF receptor 1-associated death domain), which in turn binds the death domain of another cytoplasmic protein, termed FADD/MORT-1. The latter protein also contains a death effector domain (DED) motif, which binds the DED of the ICE/Ced-3 protease FLICE/MACH-1 (Caspase 8). Activation of FLICE/MACH-1 may initiate activation of a cascade of caspases, which serves as the effector system for the apoptotic destruction of the cell.

This model suggests that ligand binding to the TNF receptor is capable of activating the final death effector pathway without apparent involvement of lipid second messengers. However, recent studies demonstrated a role for ceramide in TNF-induced cell death, in some systems. In this regard, activation of the death domain system of the 55 kDa TNF and CD95 receptors has been shown to couple to ASMase [24, 53]. This notion was based on the observation that mutations in the TNF receptor death domain which abolished apoptosis also abolished ceramide generation. Further, dominant negative FADD/MORT-1 blocked ceramide generation in BJAB B lymphoma cells, but not apoptosis induced by ceramide analogs. Whether ASMase activation might couple to FLICE/MACH-1 activation is uncertain. However, Pronk

et al. [54], using peptide inhibitors of ICE/Ced-3 proteases, molecularly ordered ceramide generation downstream of an undefined CPP32-like protease during REAPER-induced apoptosis in Drosophila Schneider L2 cells. In the present studies, TNF appeared essential for ceramide generation during the evolution of the endotoxic syndrome. Whether the TNF receptor death domain adaptor protein system is involved in lipopolysaccharide-induced ceramide generation via TNFα *in vivo*, remains, however, uncertain.

Although the present studies define ceramide as critical for the induction of endothelial apoptosis by lipopolysaccharide, its precise role in signaling apoptosis is unknown. Kroemer and co-workers have provided evidence that ceramide acts upstream of mitochondria to initiate apoptosis. Ceramide, once generated, signals mitochondrial membrane permeability transition (MPT), a committed step in the apoptotic process. MPT may signal apoptosis via release of an apoptosis-initiating factor (AIF), a Z-VAD- but not DEVD-inhibitable ICE-like protease [55]. Consistent with this paradigm, Pastroini et al. [56] showed that TNFα- and ceramide-stimulated MPT was not inhibited by the protein synthesis inhibitor cycloheximide. Alternatively, ceramide-initiated MPT may involve the release of cytochrome C from mitochondria and activation of a CPP32-like protease (Caspase 3) [57-59]. Either scenario is consistent with the inhibition of ceramide-mediated apoptosis by Bcl-2 [55, 60-62]. Whether ceramide-mediated mitochondrial damage is linked to the SAPK/JNK signaling system, also reported to be critically involved in TNF-mediated apoptosis in endothelial cells [26], is unknown.

The present investigations enhance the understanding of the mechanism of the endotoxic syndrome, defining upstream elements of the lipopolysaccharide signaling system and their molecular ordering, as well as the early tissue responses that trigger its pathogenesis. The identification of biochemical pathways that signal pro- and anti-apoptosis during the lipopolysaccharide response, and the characterization of the primary tissue target for the endotoxic syndrome, provide a molecular and cellular context for testable experimental hypotheses, and a basis for developing strategies for pharmacologic intervention, with potential for clinical application. In particular, the ability of bFGF to inhibit ceramide generation suggests that treatment with bFGF may affect the progression of the lipopolysaccharide syndrome in gram-negative septicemia with evidence of rising serum TNF, or in patients already manifesting symptoms of septic shock.

Thus, the present invention provides a method of inhibiting the generation of ceramide from sphingomyelin comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal in need of such treatment. Although this method may be useful in other animals, preferably the animal is a human. Administration of basic fibroblast growth factor is well known in the art; for this purpose basic fibroblast growth factor should be administered in a daily amount of from about 0.1 mg/kg to about 100 mg/kg.

The present invention also provides a method of treating a pathophysiological state characterized by endothelial apoptosis, comprising the administration of a pharmacologically effective dose of a fibroblast-inhibiting cytokine to said animal

such as a human. Representative pathophysiological states where this method would be useful include sepsis, radiation damage, autoimmune disease and acute respiratory distress. Preferably, the basic fibroblast growth factor should be administered in a daily amount of from about 0.1 mg/kg to about 100 mg/kg.

The present invention also provides a method of treating a method of treating a pathophysiological state characterized by sepsis in an individual, comprising the administration of a pharmacologically effective dose of a fibroblast-inhibiting cytokine to said individual.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods,

procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.